treatment. Polypeptides of interest include antibodies, monoclonal and polyclonal, cytokines, growth factors, hormones, enzymes, protein or peptide ligands and the like. Polypeptides of interest for modification by hydrazone linkage or oxime linkage forming water-soluble polymer reagent molecules of the subject invention may be isolated from their natural sources, genetically engineered cells, e.g., CHO cells transformed with expression vectors for the production of EPO, or produced by various in vitro synthesis methods. A particularly preferred polypeptide for the purposes of the instant invention is EPO, and precursors, intermediates and mimetics thereof, whether human or recombinant.

While the water-soluble polymer reagents of the subject invention may be used to modify most polypeptides, it is of particular interest to modify (1) polypeptides for use as drugs, and (2) polypeptides for use in assays. Polypeptide for use in assays include specific binding proteins, polypeptides recognized by specific-binding proteins, and enzymes. By specific-binding proteins it is intended antibodies, hormone receptors, lectins, and the like.

Various polypeptides may be modified by the subject water-soluble polymer reagents and the subject methods for their use so as to be coupled to different water-soluble polymers and to differing degrees or modification. Varying parameters such as (1) the number of water-soluble polymers coupled to an individual polypeptide molecule, which will depend upon the reactivity of the derivatized mPEGs to the EPO, and the bioactivity of the resulting mPEG-EPO; e.g., reactivity from about 3-36 molecules of

mPEG/EPO (2) the molecular weight of the water-soluble polymer, e.g., 2,000-12,000 daltons (3) the structure of the water-soluble polymer, e.g., monomethoxypoly(ethylene glycol) (4) the reaction conditions under which the reaction between the water-soluble polymer reagent and the polypeptide of interest, e.g., temperature and duration, and (5) the oxidation conditions under which the polypeptide for modification is activated for covalent conjugation, e.g., periodate at a concentration in the range of 10-40 μmol/mg of protein, may influence the biological properties of the resultant water-soluble polymer modified polypeptide.

In a preferred embodiment of the invention, activation of polypeptides for covalent conjugation is performed by mixing the protein for modification with periodate (0.1-1,000 μmole/mg protein) for a period of time in the range of one minute to three days, more preferably 0.5-50 μmole periodate/mg protein, for a time period in the range of 5 minutes to 180 minutes. In a preferred embodiment of the invention, activation for conjugation is performed by mixing the protein for modification with periodate at a temperature in the range of -10°-50°C, more preferably in the range of 0°-30°C.

In a preferred embodiment of the subject invention when the protein for modification is EPO, EPO is derivatized with the compounds of formulae II-VIII, more preferably the compounds of formulae II-V, the compound of formula III, the semicarbazide, and formula II, the carboxylate hydrazide, being particularly preferred, where the water-soluble polymer P is methoxypolyethylene glycol (mPEG) and each molecule of EPO is derivatized by 3-36, more

preferably 17-25 molecules of methoxypolyethylene glycol (in the case of the semicarbazide), and more preferably 22-32 molecules of methoxypolyethylene glycol (in the case of the carboxylate hydrazide) and the mPEG used has an average molecular weight of about 5000 daltons. Preferred reaction conditions for the production of the mPEG5000 semicarbazide modified EPO are at 0-30°C, for 5 to 60 minutes, and 0.5-50 μmoles periodate/mg of EPO (with periodate as oxidizing agent). EPO for modification by the subject water-soluble polymer reagents and methods is preferably obtained from genetically engineered cells, more preferably from CHO cells genetically modified to produce EPO. By employing the preferred water-soluble polymer reagent and conditions for modifying EPO, unexpectedly prolonged biological half-life of EPO is obtained and increased hematocrit levels can be seen, for example, see Figures 2, 4 and 5.

In another preferred embodiment, EPO is derivatized with the compounds of formulae XX-XXVII, more preferably the compounds of formulae XXI, XXIV, and XXIII, wherein the water-soluble polymer P is mPEG and each molecule of EPO is derivatized by about 18-19 mPEGS, 31 mPEGS, and 17 mPEGs, respectively, for formulae XXI, XXIV and XXIII. These results were obtained when the mPEG used has an average molecular weight of about 5000 daltons. The compound of formula XXIII had a 12mPEG fraction, which had <u>in vivo</u> bioactivity comparable to 22mPEG semicarbazide and 31 mPEG carboxylate hydrazide. Reactivity (molecules of mPEG/EPO) for formulae XXI, XXIV and XXIII was in excess of that for PEG hydrazide under the same reaction conditions. Preferred reaction

conditions for the production of the mPEG 5000 oxime may require more mild oxidation conditions such as a shorter oxidizing time or lower concentrations of oxidant than with the corresponding hydrazide to produce higher in vivo bioactivity.

The subject invention also provides methods of activating polypeptides for conjugation, i.e., covalent conjugation, with the subject water-soluble polymer reagents. These methods of activating polypeptides for conjugation comprise the step of partially oxidizing the polypeptides of interest. Partial oxidation may be achieved by adding an oxidizing agent such as periodate and other oxidation agents known to those of skill in the art, or by adding an enzyme capable of catalyzing oxidation reactions on portions of the polypeptide of interest, e.g., galactose oxidase. The preferred method of partially oxidizing a polypeptide for activation for conjugation is by the addition of periodate in a concentration in the range of 0.1-1,000 μmole/mg protein, for a period of time in the range of one minute to three days, more preferably 0.5-50 μmole periodate/mg protein, for a time period in the range of 5 minutes to 180 minutes. The temperature at which the activation is performed is preferably in the range of -10°-50°C, more preferably in the range of 0°-30°C.

Salts of any of the macromolecules described herein, e.g., polypeptides, water-soluble polymers and derivatives thereof, will naturally occur when such molecules are present in (or isolated from) aqueous solutions of various pHs. All salts of polypeptides and other macromolecules having the indicated biological activity are considered to be

within the scope of the present invention. Examples include alkali, alkaline earth, and other metal salts of carboxylic acid residues, acid addition salts (e.g., HCl) of amino residues, and zwitterions formed by reactions between carboxylic acid and amino residues within the same molecule.

The mode of administration of the preparations of the invention may determine the sites and/or cells in the organism to which the compound(s) will be delivered. The compounds of the invention can be administered alone but will generally be administered in admixture with a pharmaceutical carrier or diluent selected with regard to the intended route of administration and standard pharmaceutical practice. The preparations may be injected parenterally, for example, intra-arterially or intravenously. The preparations may also be delivered via oral, subcutaneous, or intramuscular routes. For parenteral administration, they can be used, for example, in the form of a sterile, aqueous solution which may contain other solutes, for example, enough salts or glucose to make the solution isotonic.

For the oral mode of administration, the EPO compositions of the invention can be used in the form of tablets, capsules, lozenges, powders, syrups, elixirs, aqueous solutions and suspensions and the like. In the case of tablets, carriers which can be used include lactose, sodium citrate, and salts of phosphoric acid. Various disintegrants such as starch, and lubricating agents such as magnesium stearate are commonly used in tablets. For administration in capsule form, useful diluents are lactose and high molecular weight polyethylene glycols. When aqueous solutions are required for

oral use, certain sweetening and/or flavoring agents can be added.

For administration to humans in the treatment of disease states responding to EPO therapy, the prescribing physician will ultimately determine the appropriate dosage for a given human subject, and this can be expected to vary according to the weight, age and response of the individual as well as the nature and severity of the patient's disease. The dosage of the drug in pegylated form may generally be about that employed for native drug, however, it may in some cases be preferable or necessary to administer dosages outside these limits.

It is also of interest to supply the water-soluble polymer reagents of formulae I, II, III, IV V, VI, VII, VIII, and IX, and XIX, XX, XXI, XXII, XXIII, XXIV, XXV, XXVI and XXVII separately or in various combinations, in the form of a kit, so as to provide for the convenient and reproducible derivatization of polypeptides of interest. Kits of interest may contain solutions comprising the water-soluble polymer reagent of formulae I, II, III, IV, V, VI, VII, VIII, or IX, or those of formulae XIX, XX, XXI, XXII, XXIII, XXIV, XXV, XXVI, XXVII, buffers, oxidizing agents, reaction indicator compounds, protein concentration measurement reagents, e.g., for Bradford assays, and the like. Compounds included in kits are preferably provided in pre-measured portions and pre-mixed solutions so as to provide for reproducibility and minimize error. Kits also preferably contain instructions. Instructions are directed to various steps in performing the subject methods.

SYNTHESIS OF WATER-SOLUBLE POLYMER DERIVATIVES

The following syntheses of the subject compounds are exemplary and are not included for the purpose of limiting the invention. The person of average skill in the art of organic chemistry can devise variations on the exemplified syntheses.

SYNTHESIS OF HYDRAZONE FORMING m-PEG

Synthesis of mPEG-Hydrazide

There are several ways to synthesize mPEG-hydrazide. Two methods are presented.

mPEG5000-acid (20.8 g, 4 mmol) was dissolved in 30 ml dichloromethane and t-butyl carbazate (2.64 g, 8 mmol) in 15 ml dichloromethane was added followed by 1.68 g (8 mmol) dicyclohexylcarbodiimide which was dissolved in 10 ml dimethylformamide. After running the reaction over night at room temperature the reaction mixture was filtered. The filtrate was concentrated, and the resulting residue was taken up in dichloromethane. Ether was added to precipitate the mPEG-t-butyl-carbazide which was filtered and dried. The product was placed in a dicholormethane/ trifluoroacetic acid (1:1) mixture. After 40 minutes the solution was concentrated, redissolved in dicholormethane and ether was added. The product was recovered by filtration. Yield 17.7 g. IR: (C=O) 1730, 1700. Analysis. Calcd. for N, 0.55. Found: N, 0.44.

An alternative method shown below converts mPEG-alcohol to an ester. The mPEG-ester then is hydrolyzed with hydrazine to give mPEG-hydrazide. The synthesis of mPEG-ester is similar to the procedure of Royer, G.P., and Anantharmaiah, G.M. (1979) J. Amer. Chem. Soc. 101, 3394-3395.

$$mPEG\text{-}OH \xrightarrow[Br\text{-}CH_2\text{-}CO_2C_6H_5]{NaH} mPEG\text{-}O\text{-}CH_2\text{-}CO_2C_6H_5$$

$$mPEG\text{-}O\text{-}CH_2\text{-}CO_2C_6H_5 \xrightarrow{H_2NNH_2} mPEG\text{-}O\text{-}CH_2\text{-}CO\text{-}NHNH_2$$

In a typical synthesis mPEG-OH was dried for about five hr at 85° in a high vacuum oven. After cooling 5 g mPEG-OH (MW = 5000, 1 mmol) was dissolved in 5 ml dry tetrahydrofuran. To 26.4 mg (1.1 mmol) sodium hydride was added 1 ml dry tetrahydrofuran. The mPEG5000 solution was added to the NaH dropwise. The mixture was stirred for one hr at room temperature in an argon atmosphere. During this time the solution became orange in color. Bromoacetyl acid benzyl ester (2.29 g, 10 mmol) was dissolved in 1 ml dry tetrahydrofuran, and this solution was added dropwise to the mPEG5000 mixture. The reaction was stirred overnight at room temperature under an argon atmosphere after which time it was filtered. Cold ether was added to the filtrate to precipitate the mPEG5000-benzyl ester, and the solid was collected and dried. Yield 4.5 g. IR (C=O) 1752. The compound was further purified by gel filtration on a LH-20 column eluting with methanol/methylene chloride (5:1).

The mPEG5000-benzyl ester was converted to the hydrazide by treatment with hydrazine. In a typical experiment 1.0 g mPEG5000-benzyl ester (0.194 mmol) was dissolved in 3 ml methanol/methylene chloride (5:1) in an argon atmosphere. Hydrazine (0.091 ml, 2.91 mmol) was added and the solution was stirred at room temperature for around 70 hr. The mixture was placed on a LH-20 column eluting with methanol/methylene chloride (5:1). The

mPEG5000-hydrazide was separated from the hydrazide and was precipitated with ether. The solid was collected by filtration. Yield 0.78 g. IR (H_2N-C=O): 1669. Analysis. Calcd. for N, 0.55. Found: N, 0.34.

Also synthesized were mPEG2000-hydrazide, mPEG6000-hydrazide, mPEG8500-hydrazide, and mPEG12000-hydrazide using the procedures described above.

Synthesis of mPEG-Hydrazine Carboxylate

$$\text{mPEG-O-CO-Im} \xrightarrow{H_2NNH_2} \text{mPEG-O-CO-NHNH}_2$$

The above mPEG5000-hydrazine carboxylate was synthesized as follows. Methoxypolyoxyethylene imidazolyl carbonyl (from Sigma Chemical, 2.5 g, 0.49 mmol) was treated with hydrazine (0.077 ml, 2.45 mmol) in 10 ml methylene chloride. After 4 hr at room temperature the reaction mixture was filtered, and the filtrate was treated with cold ether. The resulting precipitate was collected. Yield 2.22 g. IR (C=O): 1718. Analysis. Calcd. for N, 0.55. Found: N, 0.595.

Synthesis of mPEG-Semicarbazide

$$\text{mPEG-NH}_2 \xrightarrow{COCl_2} \text{mPEG-NH-CO-Cl}$$

$$\text{mPEG-NH-CO-Cl} \xrightarrow{H_2NNH_2} \text{mPEG-NH-CO-NHNH}_2$$

The above mPEG5000-semicarbazide was synthesized as follows. mPEG5000-amine was synthesized as described by Rajasekharan Pillai, V.N., and Mutter, M. (1980) J. Org. Chem. 45, 5364-5370. The mPEG5000-amine (2 g, 0.4 mmol) was dissolved in 9 ml dichloromethane and 0.28 ml triethylamine was added. To the mixture in an argon atmosphere was added phosgene (in toluene, 0.42 ml, 0.8 mmol). The reaction went overnight and then was bubbled with argon to remove any excess phosgene. The solution was concentrated, and the residue was dissolved in dicchloromethane and 0.063 ml hydrazine (2 mmol) was added followed by 2 ml methanol. The reaction went for 4 hr after which time cold ether was added, and the precipitate was removed by filtration and dried. Yield 1.51 g. IR (C=O): 1683. Analysis. Calcd. for N, 0.83. Found: N, 0.56.

Also synthesized were the semicarbazides of mPEG2000, mPEG6000, mPEG8500, and mPEG12000 using the procedures described above.

Synthesis of mPEG-Thiosemicarbazide

To 1.5 g mPEG5000-amine (0.3 mmol) in 5 ml dichloromethane was added 0.1 ml triethylamine (0.75 mmol) and 0.071 g (0.3 mmol) di-2-pyridylthionocarbonate. The reaction went overnight, where upon 0.047 ml (0.3 mmol) hydrazine was added. After 4 hr the mixture was filtered, and the filtrate was treated with cold ether. The product was collected by filtration. Yield 1.34 g. IR (N-H stretch): 3332. Analysis. Calcd. for N, 0.83. Found: N, 0.255.

Synthesis of mPEG-Carbonic Acid Dihydrazide

In a reaction flask purged with argon was added t-butyl carbazate (0.04 g, 0.3 mmol) in 2 ml dichloromethane, 0.084 ml triethylamine (0.6 mmol), and 0.03 g triphosgene (0.1 mmol). After 5 minutes 1.5 g mPEG5000-amine (0.3 mmol) in 4 ml dichloromethane was added. The reaction went overnight after which time cold ether was added to precipitate the product. The product was isolated by filtration. Yield 1.44 g. The protected dihydrazide (0.61 g) was treated with 2 ml trifluoroacetic acid at room temperature for 10 minutes. The trifluoroacetic acid was removed, and the resulting oil was dissolved in methylene chloride and concentrated. This step was repeated. The oil was dissolved in methylene chloride and the product was precipitated with cold ether. The product was isolated by filtration. Yield 0.41 g. IR (C=O):1695. Analysis calcd. for N, 1.37. Found: 0.41.

Synthesis of mPEG-Arylhydrazide

$$mPEG\text{-}NH_2 \xrightarrow{Boc\text{-}NHNH\text{-}C_6H_4\text{-}COOH} mPEG\text{-}NH\text{-}CO\text{-}C_6H_4\text{-}NHNH_2$$

The above mPEG5000-arylhydrazine was synthesized as follows. Boc-NHNH-C_6H_4-COOH was prepared by reacting 4-hydrazinobenzoic acid with di-tert-butyl pyrocarbonate in dioxane in the presence of base at 0°C. The protected aryl acid hydrazine (0.378 g, 1.5 mmol) was reacted with mPEG-amine (1.5g, 0.3 mmol) in a dichloromethane/dimethylformamide solution (4ml, 1:1). Also added was dicyclohexylcarbodiimide (0.31g, 1.5 mmol), 1-hydroxybenzotriazole (0.2g, 1.5

mmol), and triethylamine (0.21ml, 1.5mmol). The reaction went overnight, after which time the contents were filtered. The filtrate was treated with ether, and the precipitate was collected. The precipitate was treated with trifluoroacetic acid, and after 30 min, the trifluoroacetic acid was removed. Ether was added to the oily residue to precipitate the mPEG-arylhydrazine product. The product was isolated by filtration. Yield 1.19g. IR (N-H): 3267; (C=O): 1655; (C=C): 1606. Analysis. Calcd. for N, 0.82. Found: N, 0.50.

SYNTHESIS OF OXIME-FORMING m-PEG

Synthesis of $CH_3O-(CH_2CH_2O)_n-CH_2CH_2-CO-ONH_2$

$$mPEG-CO-NHS \xrightarrow[2.\ TFA]{1.\ BocNHOH/TEA} mPEG-O-CH_2CH_2-CO-ONH_2$$

mPEG-5000-succinimide ester (NHS) (2.0g, 0.4 mmol) was dissolved in 10ml dichloromethane. t-Butyl N-hydroxycarbamate (0.53g, 4 mmol) was added followed by 0.7ml triethylamine (5 mmol). After running the reaction overnight cold ether was added, and the resulting precepitate was collected by filtration, washed, and dried. The product (1.5g) was placed in a dicholormethane/trifluoroacetic acid (1:1) mixture. After 60 minutes the solution was concentrated. The compound was further purified by gel filtration on a LH-20 column eluting with methanol/methylene chloride (5:1). Yield 1.2g IR: (C=O) 1741. Analysis Calcd. for N,0.28. Found: N,0.13.

Synthesis of $CH_3O-(CH_2CH_2O)_n-CH_2CH_2-O-CO-ONH_2$;

$$mPEG\text{-}O\text{-}CO\text{-}Im \xrightarrow[2.\ TFA]{1.\ BocNHOH/TEA} mPEG\text{-}O\text{-}CH_2CH_2\text{-}O\text{-}CO\text{-}ONH_2$$

mPEG-5000-oxycarbonylimidazole (2.0g, 0.4 mmol) was dissolved in 10ml dichloromethane. t-Butyl N-hydroxycarbamate (0.53g, 4 mmol) was added followed by 0.7ml triethylamine (5 mmol). After running the reaction overnight cold ether was added, and the resulting precipitate was collected by filtration, washed, and dried. The product (1.5g) was placed in a dichloromethane/trifluoroacetic acid (1:2) mixture. After 60 minutes the solution was concentrated. The compound was further purified by gel filtration on a LH-20 column eluting with methanol/methylene chloride (f:1). Yield 1.2g. IR:(C=O) 1692. Analysis Calcd. for N, 0.28. Found: N, 0.15.

Synthesis of $CH_3O\text{-}(CH_2CH_2O)_n\text{-}CH_2CH_2\text{-}NH\text{-}CO\text{-}ONH_2$

$$mPEG\text{-}NH_2 \xrightarrow[2.\ BocNHOH/TEA\ \ 3.\ TFA]{1.\ Im\text{-}CO\text{-}Im} mPEG\text{-}O\text{-}CH_2CH_2\text{-}NH\text{-}CO\text{-}ONH_2$$

mPEG-5000-amine (2.0g, 0.4 mmol) was dissolved in 10ml chloroform along with carbonyldimidazole (o.23gm, 1.45 mmol). This reaction followed the procedure for activation of mPEG-alcohol with carbonyldimidazole as described by Ranucci, E., and Feruti, P. (1991) Macromolecules 24, 3747-3752. The reaction was stirred for two hours at room temperature after which time 7ml water was added, and the organic layer was extracted. The water extraction was repeated five times. The organic layer was dried over sodium sulfate, and the salt was

filtered. Added to the filtrate was t-butyl N-hydroxycarbamate (0.53g, 4 mmol) along with 0.7ml triethylamine (5 mmol). The reaction was stirred overnight after which time cold ether was added, and the resulting precipitate was collected by filtration, washed, and dried. The product (1.5g) was placed in a dicholormethane/trifluoroacetic acid (1:2) mixture. After 60 minutes the solution was concentrated. The compound further purified by gel filtration on a LH-20 column eluting with methanol/methylene chloride (5:1). Yield 1.3g. IR: (C=O):1726. Analysis. Calcd. for N, 0.55. Found: N, 0.43.

Synthesis of $CH_3O-(CH_2CH_2O)_n-CH_2CH_2-NH-CS-ONH_2$;

$$mPEG-NH_2 \xrightarrow[2.\ BocNHOH/TEA\ \ 3.\ TFA]{1.\ (C_5H_4NO)_2CS} mPEG-O-CH_2CH_2-NH-CS-ONH_2$$

mPEG-5000-amine (1.5g, 0.3 mmol) was dissolved in 30ml dichloromethane. Triethylamine (0.1ml, 0.75 mmol) was added followed by di-2-pyridylthionocarbonate (0.082g, 0.35 mmol). The reaction was stirred for two hours at room temperature after which time t-butyl N-hydroxycarbamate (0.53g, 4 mmol) was added along with 0.5ml triethylamine (3.75 mmol). The reaction was stirred overnight after which time cold ether was added, and the resulting precipitate was collected by filtration, washed, aNd dried. The product (1.6g) was placed in dicholormethane/trifluoroacetic acid (1:1) mixture. After 30 minutes the solution was concentrated. The compound was further purified by

gel filtration on a LH-20 column eluting with methanol/methylene chloride (5:1). Yield 0.72g. IR: (C=S) : 1684. Analysis. Calcd. for N, 0.55. Found: N, 0.30.

Synthesis of $CH_3O-(CH_2CH_2O)_n-CH_2CH_2-ONH_2$

$$mPEG\text{-}O\text{-}Trs \xrightarrow[2.\ TFA]{1.\ BocNHOH/TEA} mPEG\text{-}O\text{-}CH_2CH_2\text{-}ONH_2$$

mPEG-5000-tresylate (2.0g, 0.4 mmol) was dissolved in 10ml dichloromethane to which t-butyl N-hydroxycarbamate (0.53g, 4 mmol) and 0.7ml triethylamine (5mmol) were added. The mixture was heated to 45° (reflux), and the reaction ran overnight. Cold ether was added to the reaction mixture, and the resulting precipitate was collected by filtration, washed, and dried. The product (1.5g) was placed in a dicholormethane/trifluoroacetic acid (3:7) mixture. After 60 minutes the solution was concentrated. The compound was further purified by gel filtration on a LH-20 column eluting with methanol/methylene chloride (5:1). Yield 1.6g. Analysis. Calcd. for N, 0.28. Found: N, 0.19.

An alternative synthesis is the following. t-Butyl N-hydroxycarbamate (0.53g, 4 mmol) was placed in 1ml tetrahydrofuran followed by NaH (78 mg, 3.25 mmol). After a few minutes this solution was added to mPEG-5000-tresylate (1.0g, 0.2 mmol) which was in 5ml tetrahydrofuran. The mixture was heated to 40°, and the reaction went overnight. Cold ether was added to the reaction mixture, and the resulting precipitate was collected by filtration, washed and dried. the product (1.5g) was placed in a